Chapter 5

Olfactory stimulation alters the OR repertoire.

I have previously shown that the proportion of OSNs expressing any given OR is conserved between animals of the same genetic background, but differs considerably when genetic variation is introduced. Also, I have demonstrated that the observed changes are due to *cis*-acting regulatory elements and that the olfactory environment has very little effect on the WOM transcriptome. However, several studies have shown that neurones that are activated by their cognate ligands have increased life-spans[286, 288, 292]; with time, their longer survival rates translate into an enrichment in the neuronal population, compared to those OSN types that are mostly inactive[292]. Thus, one might expect that the OSNs that express receptors responsive to the odorants that are differentially produced by B6 or 129 animals, or by males and females, should be overrepresented in one strain or sex. But my results in Chapter 2 and 4 show only a few OR genes are differentially expressed in any of these comparisons. However, it is also well known that persistent exposure to any given odorant results in adaptation, where OSNs are desensitised and inhibit their responses to such olfactory stimuli[245]. Hence, could it be that the lack of DE OR genes between males and females, or between B6 animals living in a 129 olfactory environment, is the result of olfactory adaptation?

5.1 Acute but not chronic odour exposure affects OR expression levels in the WOM.

To investigate if the overall OR expression in the WOM is susceptible to change upon olfactory stimulation, I designed a set of odour exposure experiments. I chose four different odorants for which receptor-ligand interactions have been well characterised: *Olfr50* (*I-D3*) responds to (R)-carvone[224], *Olfr151* (*M71*) to acetophenone[216], *Olfr2* (*I7*) to heptanal[224] and *Olfr73* (*mOR-EG*) to eugenol[215] (Figure 5.1A). These four compounds were dissolved in mineral oil in equimolar proportions, for a final concentration of 1mM each. The odour mixture was used to stimulate B6 male and female mice, with two different presentation paradigms: 1) A *chronic* exposure, that consisted of adding the mixture to a cotton ball placed inside a tea strainer, and left in the animal's cages 24 hours a day; the mixture was replaced fresh every day. 2) Or an *acute* exposure design, where the mixture was added to the drinking water supplied to the animals (Figure 5.1B). In the chronic paradigm, the odorants were present in the environment uninterruptedly, while in the acute set-up, the animals could smell the mixture only when they approached the bottle to drink. For both experiments mineral oil was used as a control.

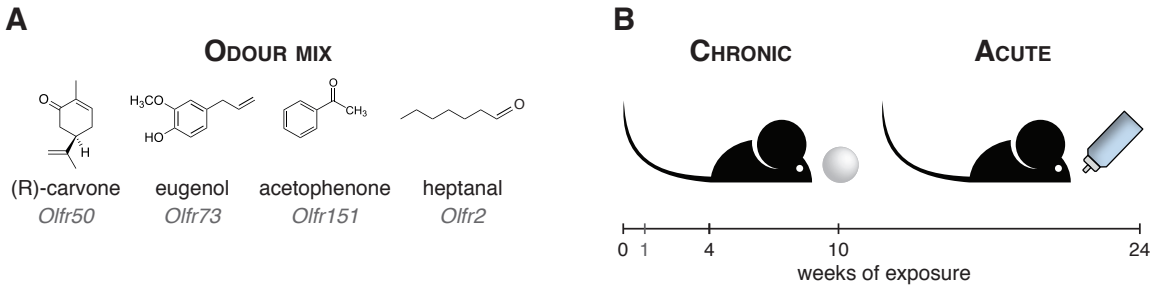

Figure 5.1 – Odour exposure experimental set-up. A) Four different odorants were used to stimulate B6 mice, as an equimolar mixture: (R)-carvone, eugenol, acetophenone and heptanal. The chemical structure of each is shown, along with one OR they activate. **B)** The odour mix was presented chronically, by applying it to a cotton ball that was left inside the mice cage 24-hrs a day; or acutely, by adding the odour mixture into the drinking water. In the acute paradigm, the animals smell the odorants only when they approach the bottle to drink. For both experiments, WOM was collected after 4, 10 and 24 weeks from the start of the treatment. For the acutely exposed animals, a further 1-week time point was included.

The odour-exposure was started from birth. I then dissected the WOM at different time-points, and assessed the expression of the OR genes expected to respond to the individual odorants, by TaqMan qRT-PCR. As *Olfr151* is a pseudogene in the B6 genome it was not included in the analysis. When the odour mixture was presented in an uninterrupted manner, no changes could be detected in the overall WOM expression of any of the three receptor genes in the odour-exposed animals compared to controls (Figure 5.2). However, the animals that were exposed acutely to the odorants, showed a

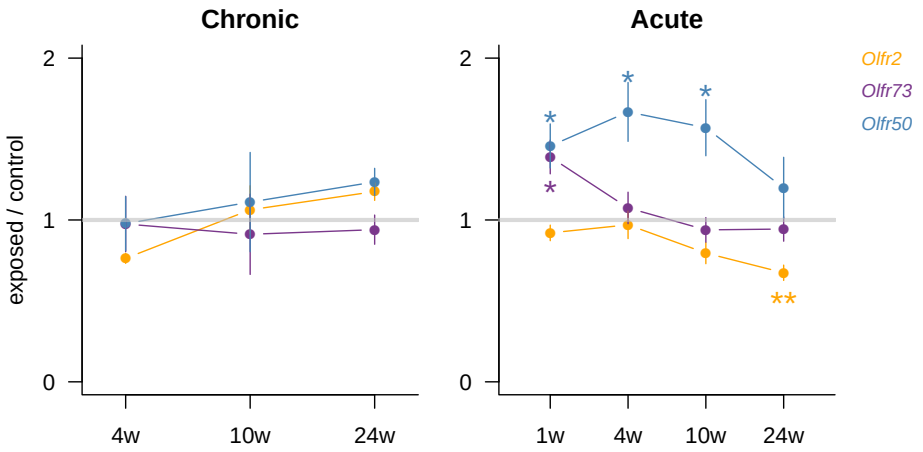

Figure 5.2 – OR expression is altered with acute stimulation. Expression estimates obtained with TaqMan qRT-PCR assays for *Olfr2* (orange), *Olfr73* (purple) and *Olfr50* (blue) at different time-points (in weeks). The ratio between the odour-exposed and the control animals is plotted. For the group exposed chronically (left) to the odour mixture, no changes were detected for any of the genes at any point. In contrast, the animals exposed acutely (right) showed significant changes for several genes. Error bars are the SEM. * P<0.05 ** P<0.01 (y-yesy, FDR < 5%). Chronic: n = 3-5 animals per group, for 4 and 10 week time-points; 9-10 for 24 weeks. Acute: n = 8-13 animals per group, per time-point.

consistent and significant upregulation of *Olfr50* at all time-points except at 24 weeks. Also, *Olfr2* was significantly downregulated at the latest time-point tested, and *Olfr73* was upregulated very early but this change was not maintained in later stages (Figure 5.2). Thus, these data suggest that OR gene expression is susceptible to dynamic change by exposure to specific odorants, but only when the stimulation is intermittent. However, it is also possible that the stimulation achieved by delivering the odorants in the water is substantially different to the chronic exposure paradigm, not only in frequency but also in intensity.

To assess the proportion of the OR repertoire that was affected by the acute exposure treatment, I sequenced RNA from six control and six experimental samples from the 24-week cohort (Tables B.1 and B.2 in Appendix B). Differential expression analysis revealed 36 OR genes were significantly regulated (FDR < 5%) upon exposure to the odour mix, with similar numbers of receptors more or less abundant in the treated animals (Figure 5.3). Most of the DE genes had small fold-changes; only one third showed differences in expression of 1.5 fold or more.

To further validate these changes, I selected seven genes with the biggest differences for which specific TaqMan probes were available, and tested their expression in all the sequenced samples plus 3 extra controls and 7 additional exposed animals. All the tested genes were statistically significant (t-test, FDR < 5%) and the direction of the changes was concordant with the RNAseq data (Figure 5.4). I then tested these genes in the

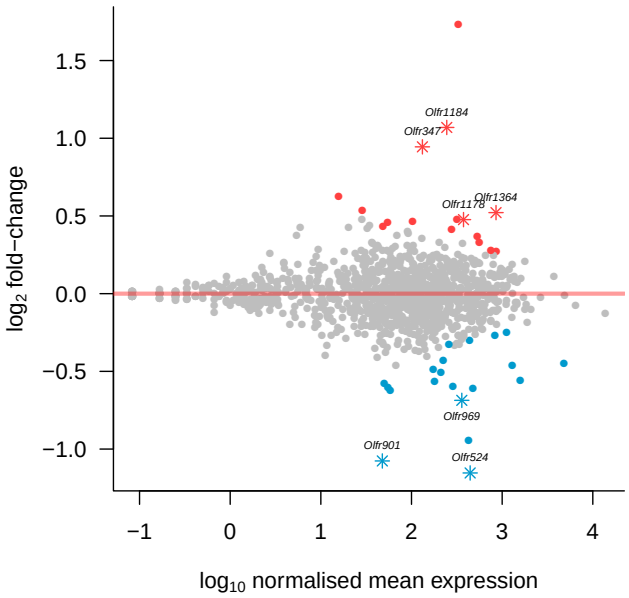

Figure 5.3 – DE OR genes in acutely exposed mice. Mean normalised expression estimates for the OR repertoire are plotted aginst their fold-change in the animals exposed acutely to the odour mix (for 24 weeks) versus controls. In all, 36 receptors are significantly differentially expressed (FDR $< 5\%$), with 16 being more (red) and 20 less abundant (blue) in the treated animals. The red line indicates equal expression in both groups. Several DE genes were selected for further validation; these are plotted as stars and their gene name is indicated. $n = 6$ animals per group.

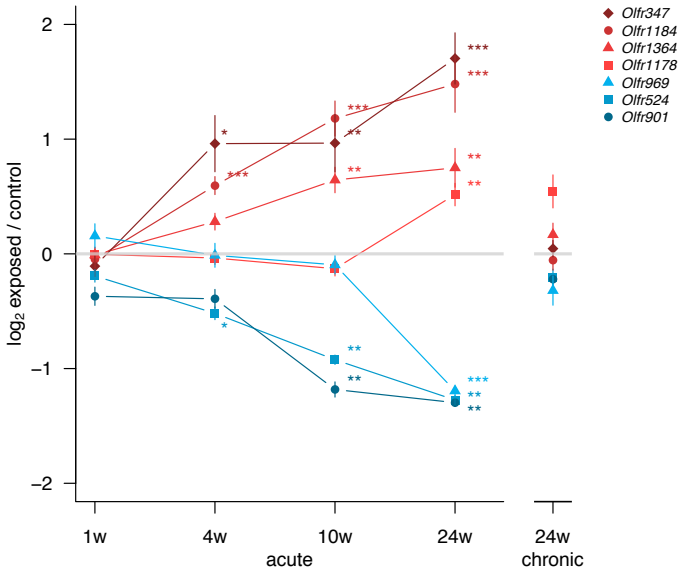

Figure 5.4 – ORs regulated by odour stimulation change in a time-dependent manner. Fold-change of the expression of seven DE OR genes in odour-enriched animals versus controls, assessed by TaqMan qRT-PCR. The ratio between groups is presented for each gene at different time-points after the start of the odour exposure, for the acutely treated mice. All seven genes are significant after 24 weeks of treatment, but some are different as early as 4 weeks. To the right is also the fold change for the same genes in the animals treated chronically during 24 weeks. No significant changes were detected. Error bars are the SEM. *P < 0.05 **P < 0.01 ***P < 0.001 (t-test, FDR $< 5\%$). $n =$ 8-13 animals per group, per time-point.

samples from previous time points. After 4 weeks of exposure, three of the genes were already statistically significantly different from controls, and at 10 weeks five out of the seven receptors were clearly DE (t-test, FDR $< 5\%$). After only one week of treatment none of the genes showed significant changes, which was expected since the pups do not drink the odourised water at this stage. For five of the DE receptors, the difference in expression relative to controls increased with time, while the other two were unchanged until the 24 week stage; these might have slower change dynamics or might require a bigger change to be detectable by measuring expression levels in the WOM (Figure 5.4). Next, I tested these same DE receptors in the samples from the mice exposed chronically to the odour mixture for 24 weeks. None of the genes were significantly different in the exposed animals compared to controls (t-test, FDR $< 5\%$; Figure 5.4). Thus, I have shown that intermittent exposure to a set of odorants results in changes in the expression of several OR genes, that become accentuated with time. However, this is not observed if the odorants are constantly present in the environment, at least for these particular set of genes.

Finally, to assess the plasticity of the observed changes, I acutely stimulated a group of animals for four weeks, and then left them to recover for an additional six weeks (Figure 5.5). I then collected the WOM and tested the expression of the same seven OR genes by qRT-PCR. As seen before, after 4 weeks of exposure, three of the seven DE ORs were significantly different and these changes increased and became more significant at 10 weeks. However, in the animals that were returned to pure water for the last 6 weeks, the expression of all the receptors was not different from controls (Figure 5.5). Thus, this data indicate that the changes in expression of certain OR genes upon odour exposure are plastic and require constant stimulation to be maintained.

5.2 Differential regulation of OR genes is odour-specific.

To better understand the effects of individual odorants on the expression of the OR repertoire, I repeated the acute odour exposure experiments but supplementing the water with (R)-carvone or heptanal alone, or with the combination of both. Controls were kept with pure water. I collected the WOM after 10 weeks of exposure and tested the expression of the seven DE ORs identified previously, by TaqMan qRT-PCR. None of the genes were significantly DE in the animals exposed to (R)-carvone alone; a marginal

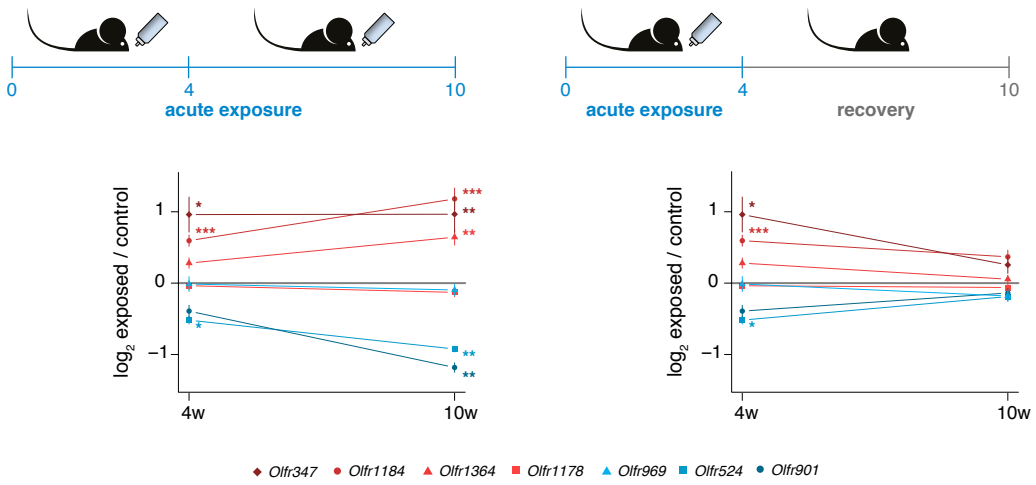

Figure 5.5 – Changes in OR abundance are plastic. Mice were acutely exposed to the odour mixture for four weeks. Then, they were returned to pure water for 6 weeks. The expression levels of the DE OR genes were tested at 10 weeks, by TaqMan qRT-PCR. The fold change between the treated and control animals are plotted for each gene. On the left is the same data from Figure 5.4; after 4 weeks of treatment three of the genes are significantly different from the controls and at 10 weeks these changes become more pronounced. On the right, the expression values returned to control levels in the animals left to recover, with no significant differences for any of them. Error bars are the SEM. *P $<$ 0.05 **P $<$ 0.01 ***P $<$ 0.001 (t-test, FDR $<$ 5%). n = 8-9 animals per group.

upregulation was observed for *Olfr50*, the carvone cognate receptor, but the difference was not significant after correcting for multiple testing. However, four of the seven tested receptor genes were significantly different in the animals exposed to heptanal, or to the combination of both odorants (t-test, FDR $<$ 5%; Figure 5.6).

To fully characterise the changes occurring in each of these experimental groups I performed RNAseq (Tables B.1 and B.2 in Appendix B). Differential expression analysis

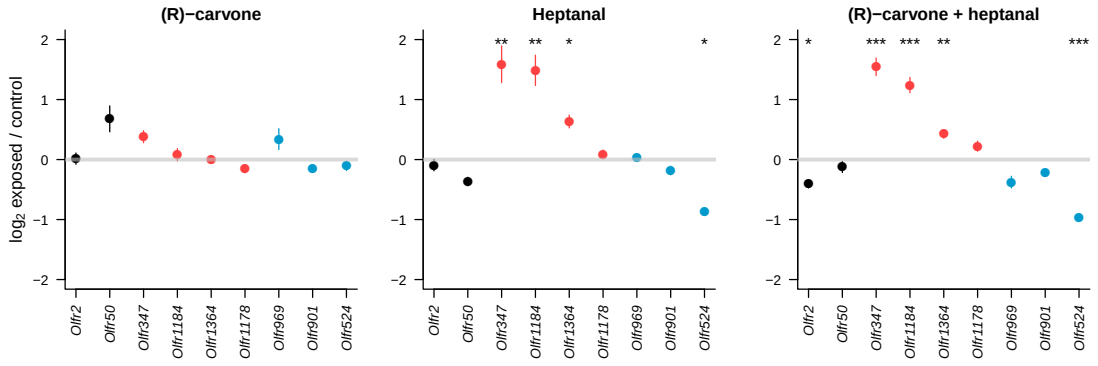

Figure 5.6 – OR genes respond to odour stimulation in a specific manner. The fold-change of the expression values for 7 OR genes previously shown to be DE upon odour-exposure are plotted for animals treated with only (R)-carvone, only heptanal or the combination of both, as assessed by TaqMan qRT-PCR. The cognate receptor for the two odorants are in black, DE genes up- and down-regulated in treated animals are in red and blue respectively. (R)-carvone has little effect on these genes and the changes are not significant. Heptanal, however, affects four of the seven genes. Error bars are the SEM. *P $<$ 0.05 **P $<$ 0.01 ***P $<$ 0.001 (t-test, FDR $<$ 5%). n = 6 animals per group.

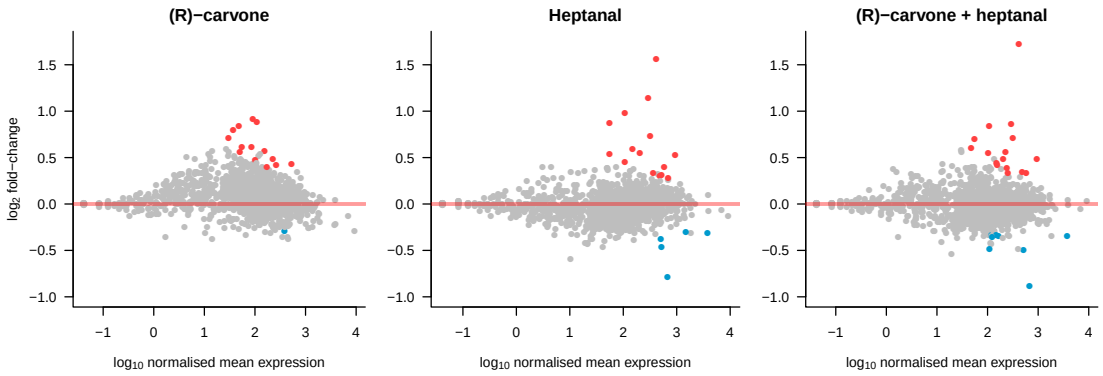

Figure 5.7 – DE OR genes in mice stimulated with different odorants. Plots of the normalised mean counts for all the OR genes versus their fold change in the experimental groups compared to controls. Each of the groups exposed to (R)-carvone, heptanal or the combination of both are presented. Statistically significant DE genes (FDR < 5%) are highlighted in red if higher in the experimental group, and in blue if lower. The red line indicates expression is not different to controls. $n = 6$ animals per group.

revealed that, in all, 43 ORs were significantly DE in at least one of the conditions (FDR < 5%), and the majority of these (74.4%) were upregulated in the odour-stimulated animals. Exposure to (R)-carvone or heptanal resulted in the change in expression of 15 and 20 OR genes, respectively (Figure 5.7). These sets of receptors were almost completely independent, with only one significantly regulated in both groups (*Olfr538*; Figure 5.8A-B). The animals that were exposed to both odorants simultaneously showed significant changes for 24 OR genes, 15 of which were shared with the individually exposed groups (Figure 5.7). Interestingly, the great majority of these overlapped with DE genes in the heptanal group, and only 2 were shared with the (R)-carvone group (Figure 5.8A). Thus, the data suggests that exposure to (R)-carvone and heptanal alters the global expression of some OR genes in the WOM, and these changes are odorant dependent. But, when both odorants are presented in combination, the effects exerted by heptanal overpower those of (R)-carvone, resulting in an expression profile that resembles more that of the heptanal group (Figure 5.8). Finally, I compared the DE ORs from both experiments, either using specific odorants or the mixture of four. Almost 40% of the ORs that showed significant changes when exposed to all four odorants were also altered in one or more of the groups exposed to (R)-carvone, heptanal or their combination.

All together, I have identified sets of OR genes that are regulated by exposure to odorants, when the stimulation is intermittent but not when the odour cues are present permanently. The receptors that are altered are dependent on the odorant used and the changes are reversible. Thus, these data are consistent with the hypothesis that constant but interleaved activation of OSNs expressing particular ORs results in a differential proportion of such OR genes in the overall WOM transcriptome. Once the stimulation

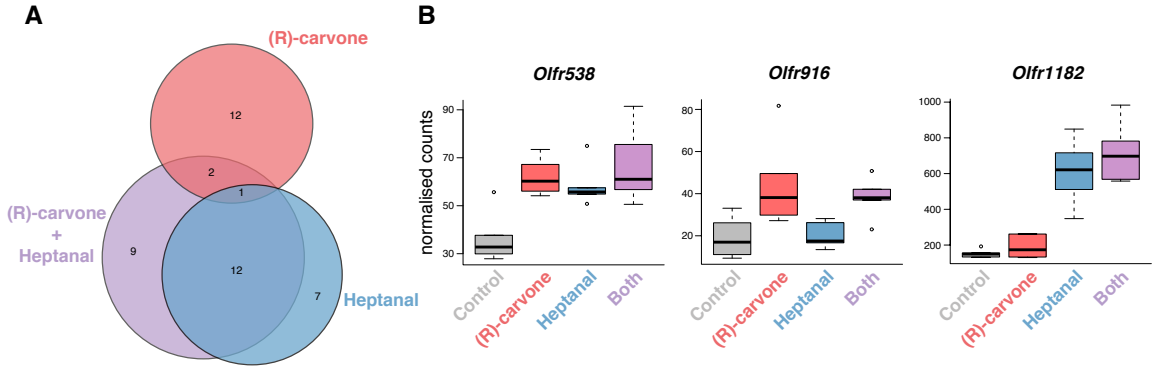

Figure 5.8 – Different ORs respond to specific odorants. A) Venn diagram showing the proportion of DE genes in each of the experimental groups exposed to (R)-carvone, heptanal or their combination (both). Genes regulated by exposure to (R)-carvone are different than those regulated by heptanal. **B)** Examples of a significant DE OR gene that is influenced by both odorants (*Olfr538*), by (R)-carvone but not by heptanal (*Olfr916*) and *vice versa* (*Olfr1182*).

ceases, the changes are reversed and the expression of the OR repertoire returns to the stable state dictated by the genetic composition of the animal.